Role of *p53* Codon72 SNP in Breast Cancer Risk and Anthracycline Resistance

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Abstract. *Background/Aim: We undertook a case-control and a case-case study to examine the possible association of p53 codon72 polymorphism with the breast cancer risk and resistance to anthracycline-based chemotherapy. Patients and Methods: Case-control study: This study enrolled 175 patients with breast cancer treated at the Salah Aziez Institute and 159 healthy Tunisian women (matched for age, ethnicity and origin), used as a control, with no clinical evidence of any neoplastic disorder. Case-Case study: 400 breast cancer patients, with invasive ductal carcinoma (IDC) treated with anthracycline based-chemotherapy. Genomic DNA was isolated from whole-blood leucocytes using the phenol-chloroform method. Anthracycline response was scored according to the World Health Organization (WHO) criteria. P53 codon72 polymorphism was genotyped using real-time polymerase chain reaction (RT-PCR) with the TaqMan method. Data were statistically analyzed using the Chi-square test. Results: Clinical data revealed that among the 400 patients, one quarter was resistant to chemotherapy treatment. Genetic data revealed that the p53 Arg72Pro genotype was found to be greatly associated with breast cancer risk ($p<0.001$), as well as tumor site ($p=0.046$). However, resistance to anthracycline-based chemotherapy does not seem to be correlated with p53 codon72 polymorphism in our population. Also, the distribution of tumor size, lymph node involvement and tumor grade was not significantly different among the polymorphic variants. Conclusion: We conclude that p53 codon72 polymorphism is involved in susceptibility to developing breast cancer. It may be a factor of progression when breast sites are taken into account. However, there is no evidence indicating that Arg72Pro SNP may influence response to anthracycline-based chemotherapy.*

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Breast cancer is the most frequent cancer in women worldwide as well as in Tunisia. Its incidence is increasing but mortality has decreased in a considerable way due to the combined effect of early detection and improvement in treatment (1, 2). One of the most effective anticancer drugs used at present are the anthracyclines. Anthracyclines are antibiotics produced from the streptomyces species (3); their general effects are believed to require a functioning apoptotic pathway to induce cell death as the primary mode of action of anthracyclines appears to be intercalation between adjacent DNA base pairs (4). Intercalation causes deformation of the DNA (5), stabilizing the normally reversible topoisomerase II-DNA complex. This stabilization results in the production of double-strand DNA breaks (6). In response to double-strand breaks in DNA, ATM (Ataxia Telanglectasia Mutated) protein kinase is activated, which, in turn, activates Chk2 (Check Point Kinase 2) kinases (7). ATM and Chk2 then both phosphorylate p53 (8, 9). Activated p53 interacts with MDM2 (Mouse Double Minute 2), which facilitates its export from the nucleus to mitochondria leading to apoptosis. Resistance to anthracycline remains the major factor limiting their use. Anthracycline resistance can arise through a number of different mechanisms, including tumor or patient characteristics, alterations in drug pharmacokinetics and metabolism and largely depends on the modification of drug target expression or function. Because anthracycline is involved in the apoptotic pathway, *p53* has been suggested to be involved in the anthracycline resistance mechanisms. Human *p53* is a tumor suppressor gene located on chromosome 17p13.1. known as "the guardian of the genome" or the cellular gatekeeper of growth and division (10, 11). The gene contains 11 exons and transcribes a 2.8-Kb mRNA translated into a 53-kDa protein (12). *p53* is a key regulator gene controlling several important cellular pathways, such as cell-cycle control, DNA repair and apoptosis. Two hundred nucleotide polymorphisms (SNPs) have been identified in the *p53* gene (13). The most investigated

Table I. *Patient and tumor characteristics*.

Variables	
Number of patients (n)	400
Median age (range) (years)	48 (20-80)
Family history of breast cancer (%)	
Yes	14
No	86
Personal history of breast cancer (%)	
Yes	8,5
No	91.5
Median age at menarche (years)	13
Marital status (%)	
Married	89
Unmarried	11
Median age at first live birth (years)	24
Menopausal status (%)	
Premenopausal	52
Postmenopausal	48
Breast (%)	
Right	44
Left	56
Histopathological type (%)	
Invasive ductal	100
Other	0
T stage (%)	
T1-T2	54
T3-T4	46
Clinical node status (%)	
N+	76
N-	24
Distant metastasis (%)	
M0	93
M1	7
Grade (%)	
I	9
II	65
III	26
HR status (%)	
Negative	29
Positive	71

Table II. *Different therapeutic settings*.

Variables	
Chemotherapeutic regimen (%)	
Adriamycin	23
Epirubicin	77
Therapeutic approach (%)	
Neoadjuvant	32
Adjuvant	63
Palliative	5
Median number of chemotherapeutic cycles n (range)	5 (1-8)
Clinical response (%)	
CR+PR	76
SD+PD	24
Anthracycline resistance	
Primary	63
Secondary	37

CR, Complete response; PR, partial response; SD, stable disease; PD, progressive disease.

Table III. *Genotypic frequency distribution in control and breast cancer group*.

Genotype	Breast cancer group n (%)	Control group n (%)	*p*-Value
Arg/Arg	19 (11%)	69 (43%)	
Arg/Pro	62 (35%)	71 (45%)	0.001
Pro/Pro	94 (54%)	19 (12%)	

polymorphism is rs1042522 (National Center to Biotechnology information single-nucleotide polymorphism (SNP) identification number), a G to C transversion (CGC to CCC) in codon 72 of exon 4, which results in amino acidic change from arginine to proline (TP53 Arg72Pro). The two isoforms differ in biochemical and biological properties. Apparently, the Arg 72 form induces apoptosis more efficiently than the pro72 form (12). One source of this enhanced apoptotic potential is the greater ability of the Arg 72 variant to localize to the mitochondria. The two polymorphic variants of *p53* are functionally distinct and these differences may influence treatment (14, 15). It has been reported that in addition to treatment response, *p53* codon72 SNP could also have an influence on cancer risk. Thus, our present project is designed to find a possible association between *p53* codon72 polymorphism with breast cancer risk and anthracycline-based chemotherapy resistance. The distribution of its alleles, in relation to many clinical parameters of the cancer group, was also investigated.

Patients and Methods

Patients. Case-control study. This study enrolled 175 patients with breast cancer treated at the Salah Azeiz Institute. The average age at diagnosis was 47 years (ranging from 22 to 80). The control group concerned 159 healthy Tunisian women (matched for age, ethnicity and origin) with no clinical evidence of any neoplastic disorder. The mean age of the control group was 46 years (range=20-93). Case-case study: Between January and June 2013, we enrolled 400 histologically confirmed breast cancer patients treated at our institute (Salah Azaiz Institut). Age at diagnosis, family and personal history of breast cancer, age at menarche, marital status (married or unmarried), age at first live birth, menopausal status, tumor characteristics, chemotherapeutic agents used, number of cycles given and response to chemotherapy were evaluated by reviewing medical files.

Evaluation of chemotherapy response. The size of primary breast tumors was determined immediately before administration of each cycle of chemotherapy and before surgery. Clinical response was

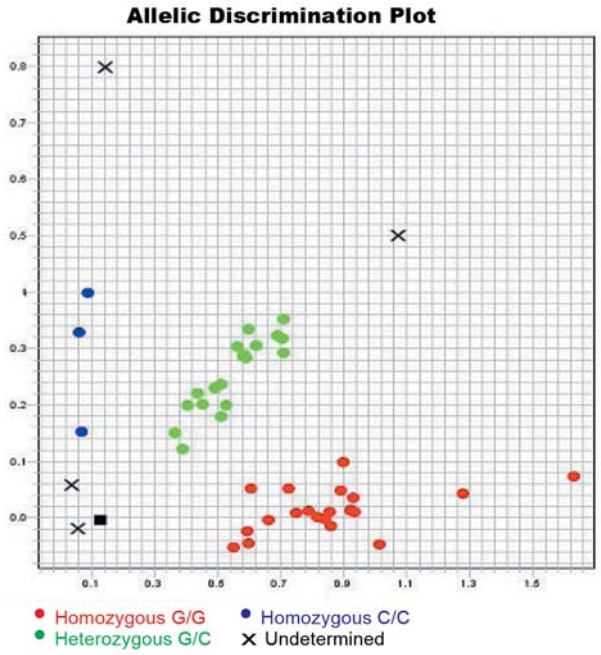

Figure 1. *Detection of the p53 codon72 polymorphism by real-time PCR "TaqMan" assay.*

scored according to the World Health Organization (WHO). In the absence of clinical evidence of tumors in the breast, response to therapy was categorized as complete clinical response (CR). Clinical response was scored as partial remission (PR) if the reduction of tumor volume exceeded 50%. Tumor less than 50% or increase of volume up to 25% was scored as stable disease (SD). An increase of more than 25% was designed as progressive disease (PD) (16).

Definition of objective response. A complete response included the disappearance of all measurable and assessable disease with a decrease greater than or equal to 50% of measurable lesions with no progression of assessable disease and no new lesion. Responders combined patients who achieved complete response or partial response (17).

Definition of anthracycline resistance. Various definitions of anthracycline resistance have been used in previous reports (18, 19, 20). In the present study, resistance was defined as disease progression during therapy or disease recurrence within 9 months of adjuvant or neoadjuvant chemotherapy with anthracycline. In our study, we defined 2 subgroups of patients with anthracycline-resistant disease. Primary anthracycline resistance was defined as progressive disease while receiving neoadjuvant, first- or second-line anthracycline-containing chemotherapy. Secondary resistance was defined as initial response followed by progressive disease (recurrence or metastases) within 9 or even 12 months after completion of neoadjuvant or adjuvant therapy or first-line containing chemotherapy for metastatic disease.

Genotyping of p53 polymorphism. Five milliliters of venous blood was collected in a sterile tube containing EDTA and stored at –80˚C. Genomic DNA was isolated form leucocytes using the phenol-chloroform method (21) and stored at 4˚C until use. Concentration and purity of the DNA were verified by a spectrophotometer (SINNOWA ER500). The absorbance ratio at 260/280 nm of all the samples ranged from 1.8 to 2 indicating they were all free from contaminants. This control enabled us to consider all DNA samples suitable for real time polymerase chain reaction (RT-PCR) assays. RT-PCR analysis was performed with Step One (Applied Biosystems, HTDS, Tunis, Tunisia). Predesigned and validated gene specific probe-based TaqMan genotyping assays form Applied Biosystems were used for the target study gene (rs1042522). Every set contained gene-specific forward 5'- AGAATGCCAGAGGCTGCTCC-3'and reverse primer 5'-GCAACTGACCGTGCAAGTCA as well as fluorescence labeled probes. Reactions were performed using the TaqMan Universal PCR Master Mix (HTDS, Tunis, Tunisia) and each reaction was plated into 48-well plates. The amplification profile was one cycle of denaturation for 30 s at 60˚C followed by 40 cycles with 15 s at 95˚C and annealing extension for 1min at 60˚C.

Table IV. *Distribution of TP53 genotypes and allele frequencies of codon72 polymorphism in breast cancer patients.*

	Genotypes			Alleles	
	Arg/Arg	Arg/Pro	Pro/Pro	Arg	Pro
Chemoresistant	15	37	43	67	123
(n=95) %	16%	39%	45%	35%	65%
Chemosensitive	34	142	129	214	396
(n=305) %	11%	47%	42%	35%	65%

Statistical analysis. Statistical analyses were performed using the IBM SPSS statistics, version 20.0 (Spss Inc, Chicago, Il, USA). Data are presented as n (%) or values. The association between the *p53* codon72 polymorphism and breast cancer risk was measured by Odds Ratios (OR) with 95% confidence intervals (CI). A comparison between the 2 breast cancer subgroups was performed using the Chi-Square test. Values of *p* equal to or less than 0.05 were considered statistically significant and strong statistical evidence against the null hypothesis.

Results

Patients' characteristics. As listed in Table I, 400 patients with proven breast cancer were enrolled into the study. The average age of our group was 48 years (range=20-80). The most common histological type was ductal carcinoma with a low degree of differentiation, grade 2 and 3 (91%). The average tumor size at diagnosis was 6 cm. About 82% of patients had clinical lymph node involvement and over 7% had distant metastasis. Detailed information of major demographic socio-economic and cancer-related variables of patients enrolled in the study is given in Table I.

Treatment completion. All patients received anthracycline-based chemotherapy for metastatic or locally progressive breast cancer. Twenty-three percent of patients received

Adriamycin and 77% received epirubicin. Patients received a maximum of 8 cycles of chemotherapy; the median number of cycles received were 5 (range=1-8). Patients were divided according to the type of therapeutic approach into the neoadjuvant (32%), adjuvant (63%) and palliative (5%) group. The reason for randomizing patients according to therapeutic approach was not to compare response between the therapeutic options.

Response to anthracycline. Clinical and pathological response data were available for 400 patients. Response to chemotherapy was assessed after all courses of anthracycline or one course in the case of clinical evidence of progression. Clinical data revealed that among 400 patients, one quarter (24%) was resistant to anthracycline-based chemotherapy. Within the study population, we defined 2 sub-groups of patients with anthracycline-resistant disease. Sixty-three percent of patients had primary anthracycline resistance and 32% had secondary anthracycline resistance (Table II).

Genotyping. Association between p53 codon72 polymorphism and breast cancer risk (Figure 1). The frequencies of the genotypes and alleles in the *p53* gene are shown in Table III. The frequencies of Arg/Arg, Arg/Pro and Pro/Pro were found to be: 11% (19/175), 35% (62/175) and 54% (94/175) in the breast cancer cases and 43% (69/159), 45% (71/159) and 12% (19/159) in the controls, respectively. All the results were in Hardy-Weinberg equilibrium. This result shows that the Pro/Pro genotype is more prevalent in breast cancer patients, while the Arg/Arg and Arg/Pro are more abundant among normal controls. By comparing the statistical genotype Pro/Pro with the two other genotypes in both groups, there was a statistical meaningful difference between control group and breast cancer group (p<1%, OR=8.55, IC=4.737–15.862) suggesting that the Arg/Arg and Arg/Pro genotypes had an association with protection against breast cancer, whereas the Pro/Pro was associated with breast cancer in the Tunisian population.

Association between p53 codon72 polymorphism and response to anthracycline-based chemotherapy. When the genotype frequencies corresponding to *p53* polymorphism were compared between chemosensitive and chemoresistant patients, no statistically significant differences were observed. The genotypic distributions of the 2 groups are presented and compared in Table IV. Neither of the alleles of *p53* polymorphism were found to be associated with resistance to anthracycline ($p>0.05$). The percentages of allele G and C among chemosensitive and chemoresistant patients were 35% and 65%, respectively. We also evaluated the relationship between SNP72 Arg/Pro of *p53* and the clinicopathological factors of breast cancer. The distribution of tumor size, lymph node involvement and Scarff-Bloom-Richardson (SBR) grade was not significantly different among the polymorphic variants. However, we observed a marginally significant interaction with tumor site suggesting that the Pro carrier genotypes seem to have greater possibility of having arisen from the left breast than from the right ($p=0.046$, OR=1.92).

Discussion

The p53 tumor suppressor protein is involved in multiple central cellular processes, including transcription, DNA repair, genomic stability, senescence, cell-cycle control and apoptosis (22). P53 activities are acutely related to its structure and even subtle polymorphism at the level of single nucleotide polymorphisms may exert a profound effect on its performance (23). Two hundred different SNPs in the *p53* have been reported (13). The most investigated polymorphism is rs1042522, a G to C transversion in codon 72 of exon 4, which results in an amino acidic change from arginine to proline (*p53* Arg72Pro). The rs1042522 polymorphism is located in a proline-rich region of the protein, which has been known to be important in mediating the apoptotic response (24). Apparently, the Arg72 form induces apoptosis more efficiently than the Pro72 form (14, 15). A large number of studies have investigated the role of the functional Arg72Pro polymorphism in the modulation of cancer risk. The majority of studies have reported the association of cancer risk with pro72 allele (25, 26, 27) and few with Arg72 allele (28, 29). Our findings are consistent with the model in which the p53 codon 72 Pro variant is associated with the development of breast cancer ($p=0.001$. OR=8.55, IC=4.737-15.862).The frequency of *p53* rs1042522 Arg/Arg, Arg/Pro and Pro/Pro genotypes were: 11% (19/175), 35% (62/175), 54% (94/175) in the case group compared to 43% (69/159), 45% (71/159), 12% (19/159) in the control group. These results are in agreement with other published data, which have reported a marked association between Pro/Pro form of SNP 72 and breast cancer risk (30). However, AL-Qasem *et al*., Alawadi *et al*. and a recent meta-analysis by He *et al*. showed the Arg/Arg genotype to be associated with increased breast cancer risk among the Saudi Arabia, Kuwaiti and Indian population, respectively (28, 31, 32). In contrast, other studies did not observe any association between the *p53* codon72 polymorphism and breast cancer risk (33-35). Arg72Pro SNP has also been reported to be involved in susceptibility to lung, pancreatic, esophageal, cervical, oral squamous cell, gastric and colorectal cancer (25, 29, 30, 37-39). *P53* codon 72 polymorphism may affect not only cancer development but also cancer progression. Few other studies have investigated the effect of *p53* codon 72 polymorphism in prognosis. A significantly lower overall survival rate was observed for patients with endometrial carcinoma harboring *p53* codon 72

SNP (p=0.0029) (40). In acute myeloid leukemia patients, the *p53* codon 72 SNP was associated with better median OS; patients with Arg/Arg have better median OS than the Arg/Pro and Pro/Pro patients (p=0.045) (41). Similar results have been reported in advanced head and neck cancer (23). Moreover, the *p53* codon 72 SNP was found to be correlated with the time to progression in patients with advanced gastric cancer; the time to progression for patients with Arg/Pro and Pro/Pro genotypes was worse than for the patients with Arg/Arg genotypes (p=0.047) (42). Rs1042522 was recently identified as important variant that could influence the response to cytotoxic drugs, radiation and chemoradiation both *in vitro* and *in vivo*. The *p53* codon 72 SNP was found to be predictive of the response to chemotherapy in patients with advanced gastric cancer treated with paclitaxel and cisplatin chemotherapy; the Arg/Pro and Pro/Pro genotypes were significantly correlated with a lower response rate to the combination chemotherapy when compared to Arg/Arg (42). The same effect was shown in many cell types (H1299) and cell lines of lung carcinoma (43). These findings inspired us to investigate the association between *p53* codon72 polymorphism and resistance to anthracycline-based chemotherapy in a large series of breast cancer. We used the TaqMan SNP genotyping assay, which is amenable to high throughput genotyping and avoids many problems of traditional genotyping assays, such as PCR-Restriction Fragment Length Polymorphism (44). We did not find any statistically significant association between the Arg72Pro and resistance to anthracycline. By contrast, one study on breast cancer among the Chinese population found an association between *p53* codon72 polymorphism and the pathologic response to neoadjuvant-based chemotherapy. From that study it emerged that those carrying a Pro/Pro variant may be less sensitive to anthracycline-based treatment than those with Pro/Arg and Arg/Arg (45). In the present report, the distribution of tumor size, lymph node involvement and SBR grade was no significantly different among the polymorphic variant. This is in agreement with the data of other authors, who also find no association between Arg72Pro polymorphism and clinical parameters of breast and colorectal cancer (27, 45). However Xu *et al*. found that patients with the Pro/Pro variant more frequently displayed a positive lymph node status than those with Arg/Pro and Arg/Arg (45). In contrast, Han *et al*. showed that patients with Arg/Pro or Pro/Pro variants had a negative lymph node status (46). The precise reasons for this discrepancy is that the effect of this polymorphism varies according the genetic background of the study populations. We also examined the interaction between *p53* codon72 polymorphism and tumor site. We observed a significant difference of the homozygous *p53* Pro genotype compared with combined Arg/Arg and Arg/Pro (p=0.046) suggesting that the Pro carrier genotypes had greater possibility of having breast cancer in the left than in the right site. A strong association of the *p53* codon 72 SNP and tumor location was observed in colorectal cancer (CRC); when colorectal site was accounted for the Pro carrier genotypes compared to Arg/Arg, they were associated with an increased risk of proximal colon cancer in women, while among men the same genotypes were associated with in increased risk of distal colon cancers (26).

In addition, a previous study among the Greek-Caucasian population associated the Arg/Arg genotype with an increased incidence of left colon cancer (p=0.026) (29). To conclude, our results suggest that a reduced efficiency of *p53*-induced apoptosis due to the presence of the Pro allele may affect breast cancer risk, as well as tumor site among the Tunisian population. However, there is no evidence indicating that Arg72Pro SNP of *p53* may influence response to anthracycline-based chemotherapy. Why are tumors often inherently resistant to chemotherapeutic drugs or become resistant after an initial round of treatment? In the present report, we still have limited knowledge regarding which processes may control resistance. It may be due to hypoxia because anthracycline requires oxygen to generate free radicals that contribute to toxicity. Moreover, hypoxia might modulate expression of enzymes directly involved in metabolism of anthracycline, thereby limiting its toxic effect on cancer cells. The other possibility includes the over-expression of Topo-II, which is the major target for anthracycline therapy. It is evident that anthracycline kills tumor cells by activating common apoptotic pathways; thus, inactivation of genes in the same pathway may be a mechanism causing resistance to anthracycline.

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